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IRON ACCUMULATION IN TUNICATE BLOOD CELLS. I. DISTRIBUTION AND OXIDATION STATE OF IRON IN THE BLOOD OF *BOLTENIA OVIFERA, STYELA CLAVA,* AND *MOLGULA MANHATTENSIS*

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Abstract

The iron concentration, oxidation state, and distribution in blood plasma and blood cells of three iron containing tunicates were determined. Preliminary studies are reported on the possible role of plasma proteins in iron uptake.

Iron(II) concentration in the millimolar range was found in the blood cell cytoplasm of all three species; no iron(III) in solution was detected in blood cells. Over 70% of the total iron in the cells is associated with the membranes.

Although the iron concentration in *S. clava* blood cells is substantially greater than that in *B. ovifera* cells, the iron to protein ratio by weight is similar in both species. SDS-electrophoresis of *B. ovifera* blood showed two protein subunits common to both plasma and blood cells. These two subunits are most likely the major components of the high molecular weight protein found in the plasma. This protein was shown to bind iron(III) when iron(III) citrate was added to the plasma.

Introduction

Mechanisms of metal ion transport and accumulation in living cells are now being investigated by new techniques (Marx and Aisen, 1981; Anderson and Morel, 1982), and new tools such as extended x-ray absorption fine structure, EXAFS (Tullius *et al.,* 1980). Of the essential metallic elements, iron presents one of the most difficult systems to study in terms of elementary steps at the organism/environment and cell/plasma barriers. Studies with bacteria (Emery, 1982) provide detailed information on elementary steps in uptake, although information on comparable processes in animals still remain obscure. Studies with tunicates have the potential to clarify several steps in the accumulation process.

Tunicates (class Ascidiacea) accumulate relatively high concentrations of selected metal ions in certain blood cells. Best known is the ability of members of the order Enterogona to accumulate vanadium (Millar, 1966; Swinehart *et al.,* 1974). We have identified several elementary steps in the selective vanadium uptake mechanism and a model for this process has been constructed (Dingley *et al.,* 1981). We have recently extended our investigations to include iron accumulating Pleurogona (Agudelo *et al.,* 1982; Agudelo *et al.,* 1983). In this paper we begin our analysis of the iron accumulation mechanism by detailing the distribution, concentration, and oxidation

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Abbreviations. TEMED, N,N,N′,N′-Tetramethylethylenediamine; SDS, sodium dodecyl sulfate; Bis, N,N′-Methylene-bis-acrylamide.

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state of the element in the blood of three iron-accumulating ascidians.

The iron concentration in these species is about one to two orders of magnitude less than that of the vanadium concentration in vanadium-containing tunicates. However, the iron concentration gradient is still very large, when compared with the iron in the aqueous phase of sea water. Like the Enterogona, the Pleurogona contain similar blood cell types, as well as tunichrome (Macara *et al.*, 1979).

Materials and Methods

Materials

NaCl, $BaCl_2$, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, KSCN, 1,10-phenanthroline, hydrochloric acid, acetic acid, nitric acid, glycerine, and bromophenol blue dye were purchased from Fisher Scientific Co.

Sephadex G-75 and blue dextran were purchased from Pharmacia Fine Chemicals.

Acrylamide, Tris buffer, TEMED, SDS-MW70 molecular weight markers kit, albumin total protein standards and OsO_4 were obtained from Sigma Chemical.

Bis, Coomassie brilliant blue, glycine, ammonium persulfate, and 2-mercaptoethanol were obtained from Bio-Rad Laboratories.

ACS aqueous counting scintillant was obtained from Amersham Corporation.

$^{55}FeCl_3$ was obtained from New England Nuclear.

2,2′bipyridine was obtained from Mallinckrodt and ascorbic acid from Schwarz/Mann, Inc.

All chemicals were used without further purification.

Specimens

Boltenia ovifera was collected by divers off East Point, Nahant, MA at 20 m depth. *Styela clava* was obtained from the Boston Harbor; *Molgula manhattensis* was purchased from Marine Biological Laboratory, Woods Hole, MA. Animals were all maintained in running sea water at 5–10°C. Blood of *B. ovifera* and *S. clava* was extracted as described previously (Agudelo *et al.*, 1982). Blood of *M. manhattensis* was obtained by cutting the tunic at the base of the animal and allowing the blood to drip into a test tube. Blood cell types were classified according to the criteria summarized by Wright (1981). Blood cells were fixed and stained with osmium tetroxide vapors (Kalk, 1963).

Blood cells were separated from the plasma by centrifuging at 1200 g for five minutes. Plasma was frozen for later analysis; cells were used immediately.

Iron oxidation state and concentration

For the oxidation state analysis of blood, cell samples were treated with 6*N* HCl, heated in a boiling water bath for five minutes and centrifuged at 18,400 g for twenty-five minutes. For plasma, the centrifugation step was omitted.

Total iron concentration in blood cells was determined by using a Perkin Elmer Model 305 atomic absorption spectrometer. The reduced iron concentration inside the cells was obtained by lysing the cells in 6*N* HCl and adding excess 1,10-phenanthroline. The $Fe(Phen)_3^{2+}$ absorbance was measured at 510 nm using a Perkin Elmer 552A uv/vis spectrophotometer. The molar absorptivity coefficient of the tris(1,10-phenanthroline) iron(II) complex at 510 nm, pH 1 (HCl) and room temperature was determined to be 7.6×10^3 cm^{-1} M^{-1}.

The calculation of total cell volume in blood cell samples was based on the assumption of spherical cells with mean cell diameter 16 μm, and cell counts using

a Levy-Hausser Hemocytometer. Total protein content was determined by the Lowry method (Lowry *et al.*, 1951).

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) experiments were carried out at room temperature on a previously described spectrometer (Dingley *et al.*, 1981). The fluoride method (Levanon *et al.*, 1968) was used to gain maximum sensitivity in the detection of iron(III). The spectrometer settings for this experiment were: 9.55 GHz, 9mW power, time constant 1 s., modular amplitude 5.0, gain 12.5. Under these conditions, the minimum amount of iron(III) we could detect was approximately 10 micromoles. Concentrated solutions (approximately 1.3 *M*) of ammonium fluoride (NH_4F) were added to freshly drawn samples of blood producing a dilution factor of about one-third. The final pH was 6.5. This treatment ensured that the blood cells in the EPR tube were as intact as possible, and that the large excess of fluoride would convert even tightly chelated intracellular iron in solution to the FeF_6^{3-} form.

Chromatography and electrophoresis

The water soluble proteins in the plasma and blood cell cytoplasm were run through a size exclusion chromatography column 30 cm long and 1.5 cm in diameter packed with Sephadex G-75. The eluent was 0.5 *M* sodium chloride and 0.02 *M* HCl. Absorbance at 280 nm was monitored continuously using an ISCO UA5 absorbance monitor. Fractions were collected automatically and analyzed for iron either by atomic absorption spectrometry or by adding an excess of 2,2′-bipyridine and ascorbic acid and measuring the absorbance of the iron(II)-bipyridine complex at 520 nm (Macara *et al.*, 1979). Column void volume (V_o) and bed volume (V_t) were determined using blue dextran and vitamin B_{12} respectively.

SDS-acrylamide gel electrophoresis of *B. ovifera* blood cells and plasma was carried out by the Laemmli method (Laemmli, 1970) using vertical gel slabs; β-lactoglobulin (18,400 d), trypsinogen (24,000 d), egg albumin (45,000 d), and bovine albumin (66,000 d) were used as molecular weight standards. The gels were fixed for twelve hours with 10% trichloroacetic acid, stained with 0.25% Coomassie brilliant blue dye for four hours, and destained with 7% acetic acid. Independent of the molecular weight determination, and in order to avoid denaturation, electrophoresis of the native proteins in the plasma was run excluding SDS.

Results

In the first section we report the iron concentration in tunicate blood plasma and blood cells, the oxidation state of the iron, the iron distribution in the plasma, cytoplasm, and the cell membranes (no differentiation between cell and intracellular membranes was made). Protein distribution is reported in the second section.

Iron

The oxidation state of the iron present in solution can be established before a quantitative determination of the total iron content of the plasma and blood cells is carried out. The advantage to this approach is that once the predominant oxidation state of the iron is known, more than one method for total iron determination can be employed, and the results compared.

TABLE I

Oxidation state analysis of iron obtained from blood cell cytolysis

Test reagent	Tunicate species		
	B. ovifera	*S. clava*	*M. manhattensis*
1,10-phenanthroline	+	+	+
$K_3Fe(CN)_6$	+	+	+
$K_4Fe(CN)_6$	−	−	−
KSCN	−	−	−

The results of the oxidation state analysis of the iron in the cell lysates (cytoplasm) of all three species are tabulated in Table I. The oxidation state of the iron in blood cells is found to be in the Fe(II) form as was previously reported for *Pyura stolonifera* (Endean, 1955). No precipitation occurred when barium chloride was added to blood cell lysates, indicating the absence of sulfate. After applying the same test reagents, shown in Table I, to plasma of *S. clava* acidified with concentrated HCl, we find that both iron(II) and iron(III) are present. Addition of barium chloride gave a white precipitate.

Osmium tetroxide (OsO_4) vapors were used to localize regions of the blood cell with reducing ability. As indicated by the staining results (Table II), it is concluded that most of the reducing substances are found in the vacuolated cells; *i.e.*, morula, compartment, and signet ring cells. Amoebocytes also show some staining.

Several attempts were made to detect iron(III) by the EPR method. Since the sensitivity depends on the total number of spins in the spectrometer, hence the cell count, blood samples from two or three specimens were pooled. The characteristic seven line spectrum of the FeF_6^{3-} complex was not observed in any of these experiments.

Since we found no iron(III) by the available methods, total iron concentration within the cell was determined by the 1,10-phenanthroline method, which is specific for iron(II). The accuracy of this method is limited by the accuracy of the volume determination of the blood cells based on the estimated average cell diameter and total cell count (10–15% error). To simplify calculations, it is assumed that all cell types have equal amounts of iron. Total iron concentration determination using

TABLE II

Osmium tetroxide staining of blood cells

Cell type	Tunicate species		
	B. ovifera	*S. clava*	*M. manhattensis*
Morula cell vacuoles	+++ or −*	+++	+++
Compartment cell vacuoles	**	+++	+++
Signet ring cells	+++	NI	NI
Amoebocytes	+ or −	++	+++
Lymphocytes	−	−	+ or −

* Some vacuoles are stained others are not.

** A few stained vacuoles; in general, clear vacuoles and stained cytoplasm.

NI not identified in blood smears.

TABLE III

*Iron concentration in blood cells as determined in pooled samples**

Species	Method	
	$Fe(Phen)_3^{2+}$	AA
B. ovifera	1×10^{-3} M	6×10^{-3} M
S. clava	$5–9 \times 10^{-3}$ M	7×10^{-2} M
M. manhattensis	8×10^{-3} M	—

* Relative accuracy is limited by volume determination (±10–15%) and/or iron detection.

atomic absorption spectrometry (AA) is also dependent on cell volume and cell count determination. The results for both methods are shown in Table III. The iron content of the plasma as determined by AA was 1.6–1.8 ppm for *S. clava* and *B. ovifera*.

There is an order of magnitude difference in iron concentration in the blood cells by the two methods. This difference is greater than that expected from error in cell volume and cell count determinations. Procedural differences between iron analysis methods account for this observation. In the phenanthroline method the cell membranes are discarded, while in the atomic absorption analysis the whole cells are digested and analysed. Therefore a large fraction of the iron in the cells is associated with cell membranes.

To determine how much of the iron is found in the cytoplasm and in the cell membranes, the following analysis was carried out: the blood cells were lysed with distilled water, the lysate was separated from the membranes by centrifugation at 18,400 g for 30 min. The cell membranes were then resuspended in 0.1 *N* HCl, mixed thoroughly, and separated again by centrifugation at 18,400 g for 30 min. The membranes were then digested with concentrated nitric acid for 3–4 hours until a clear solution was obtained. The results of the iron analysis by atomic absorption of the cell lysate, 0.1 *N* HCl wash and digested membranes are tabulated as percent iron in Table IV.

As shown in Table IV, over 70% of the iron is bound to the cell membranes. The 0.1 *N* HCl wash removes any iron that might have precipitated during cell lysis with distilled water, as well as any loosely bound surface iron. Since most of the iron is found in association with cell membranes, volume concentration units are illusory. Analysis of the iron content per weight of protein was therefore carried out. The results yield 0.05 ± 0.01 μg Fe/mg protein in the plasma, and 1.1 ± 0.2 μg Fe/mg protein in blood cells of *B. ovifera*. In the blood of *S. clava* we found 0.14 ± 0.1 μg Fe/mg protein in the plasma and 1.11 ± 0.05 μg Fe/mg protein in blood cells.

TABLE IV

Relative iron distribution in blood cells as determined in pooled samples

Species	Cell lysate	HCl wash	Cell membranes
B. ovifera (1)	14%	—	86%
" (2)	2%	7%	91%
S. clava	11%	16%	73%

Proteins

Size exclusion chromatography of the cell lysate (cells lysed with 0.1 *N* HCl) gave an absorbance profile with two main peaks. One peak eluted at the exclusion limit, V_o (molecular weight greater than 75,000). The second peak eluted at the bed volume, V_t (molecular weight less than 3000), and is assigned to tunichrome (Macara *et al.*, 1979), which has a lower molecular weight. Iron was eluted with both the high molecular weight protein and tunichrome fractions, some iron was eluted after the tunichrome peak at a k_{ave} of 1.2.

Slightly different results were obtained in chromatography of the plasma. Only a high molecular weight protein peak was observed. In a few cases a low molecular weight peak, attributed to tunichrome, was also found, probably because of cell lysis during centrifugation. The iron concentration in the plasma is very low, 1.6–1.8 ppm, and no iron was detected in the high molecular weight protein fractions. A small amount of iron was detected at a k_{ave} of 1.2.

For a better characterization of the proteins in the plasma and the water insoluble proteins found in the cell membranes, SDS-acrylamide gel electrophoresis of *B. ovifera* blood cells and plasma was carried out using the Laemmli method. The protein subunits found in the plasma and the cell membranes are shown in Figure 1. With 10% acrylamide gel two main bands were observed for the plasma corre-

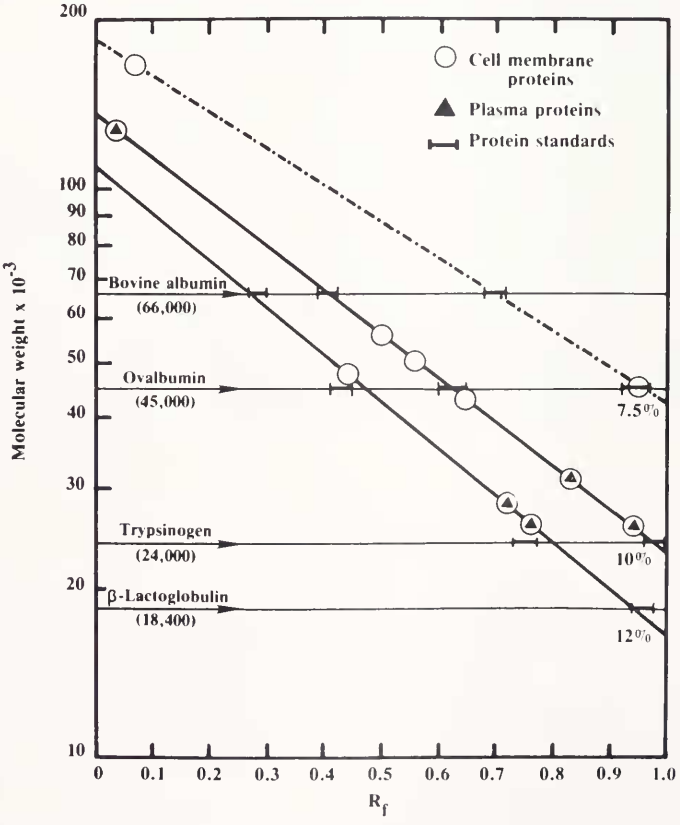

FIGURE 1. SDS-acrylamide gel electrophoresis (Laemmli method) of *B. ovifera* blood plasma (10% and 12% gels) and blood cells (7.5%, 10%, and 12% gels).

sponding to 31,000 and 26,000 d. The cell membrane samples showed a large number of proteins; only the most visible and clear bands are reported: a strong band at 130,000, two faint bands at 56,000, and 51,000; a strong band at 44,000, a medium band at 31,000, and a strong band at 26,000 d.

With 12% acrylamide gel results showed two bands for the plasma sample at molecular weights 28,000 and 26,500, which were also observed in the cell membranes. By referring to Figure 1, we see that these 12% acrylamide gel results correspond to the 31,000 and 26,000 bands in the 10% acrylamide gel experiment. These two bands are the only ones observed to occur in both the membranes and plasma. A strong band was also observed in the membrane sample at 48,000 d corresponding to the 45,000 d band observed in the 10% gel. The two faint bands observed at 56,000 and 51,000 with a 10% gel, and the 130,000 band were not observed.

To insure that the 130,000 molecular weight protein observed using a 10% acrylamide gel was not an artifact, a 7.5% acrylamide gel was run. Since no high molecular weight standards were available and our main concern was to determine the existence of a high molecular weight protein, and not its exact molecular weight, only two standards were used, bovine albumin and egg albumin. If a straight line is assumed to pass through the two molecular weight standards used when R_f values were plotted against log(molecular weight) in Figure 1, the following conclusions can be drawn. The strong band observed at an R_f of 0.07 corresponds to an approximate molecular weight of 160,000, and the strong band observed at an R_f of 0.95 corresponds to a molecular weight of 45,000.

To determine the iron binding properties of the plasma proteins, we added to 1.0 ml of *B. ovifera* blood plasma 0.050 ml of an ^{55}Fe-citrate stock solution containing 1,000-fold excess citrate to prevent Fe(III) precipitation. The sample was allowed to stand for 15 minutes at 0°C. Gel electrophoresis of the native proteins was carried out, taking care not to denature the proteins by excluding SDS from the procedure, preventing any changes in the native protein configuration that would alter its Fe-binding properties. After the electrophoretic separation of the proteins, the gel was cut vertically into two pieces. One piece was fixed and stained as described in the methods section. To prevent any radioactive iron loss into the fixative solution, the other piece was not fixed. This gel was cut into 0.5 cm horizontal sections and analysed for ^{55}Fe by liquid scintillation counting. In the stained piece we observed one band. The largest ^{55}Fe activity was observed at the R_f value corresponding to this protein band, indicating that the native plasma protein has the ability to bind iron.

Discussion

Oxidation state +2 predominates for iron found in the blood cell cytoplasm of the three ascidians *B. ovifera, S. clava,* and *M. manhattensis.* No iron in oxidation state +3 was detected. The blood plasma, however, contains both iron(II) and iron(III). This finding is not surprising; even if only iron(II) is present in the plasma, then as soon as the plasma is exposed to air, some of the iron will be oxidized to the +3 oxidation state. It is also possible that iron is in the +3 form in the plasma and is reduced as it goes into the cell. In this case, the +2 iron in the plasma arises from cell lysis, exchange, or leaching.

The iron concentration in blood cells varies from species to species, similar to the variation in vanadium concentration among vanadium-containing tunicates

(Hawkins, personal communication). Tunicates therefore accumulate iron against an approximately 10^5–10^6 concentration gradient (ratio of iron in tunicate blood cells, 10^{-3}–10^{-2} *M*, to dissolved iron in sea water, 2×10^{-8} *M* (Kester *et al.*, 1975)).

Although there is considerable iron concentration in the cell cytoplasm, a large fraction of the total iron was found in the cell membranes (over 70%). We have not yet determined the oxidation state of the membrane-bound iron, which will require more complicated techniques than those we report in this paper.

The OsO_4 staining method has often been used to determine the metal ion distribution in cells (Henze, 1913; Endean, 1960; Kalk, 1963; Fuke, 1979). However, we encountered several problems in the interpretation of this method. OsO_4 is sensitive to many strong reducing agents. Along with Fe(II), tunicate blood cells contain tunichrome, a relatively strong reducing agent (Macara *et al.*, 1979) capable of reacting with OsO_4 to generate dark stains. There is also considerable variation in the results among similar cell types. Due to these considerations, OsO_4 staining leads us to conclude that there are one or more reducing agents (iron(II), tunichrome, or both) in the morula cell vacuoles of all three species as well as in compartment cell vacuoles. The staining of *B. ovifera* compartment cell cytoplasm and not vacuoles cannot be explained easily. Leaching of the vacuolar contents during the staining procedure would result in uniform staining throughout the cell. On the contrary, the vacuoles remained intact and clear, whereas the cytoplasm was stained deeply.

Metal ion content of blood cells is often given in volume-based concentration units, such as moles/liter (*e.g.*, Tullius *et al.*, 1980). However, since most of the iron is found in the cell membranes, and is probably associated with a specific protein, we find it more useful to report the iron content as μg Fe per mg of protein. With this unit we find a smaller difference in the iron concentrations of *B. ovifera* and *S. clava;* approximately 1 μg Fe/mg of protein in the blood cells of each species. This value is comparable to that of other iron-accumulating blood cells. For example, it is comparable to the concentration of 3.48 μg Fe/mg of protein in human erythrocytes if hemoglobin is used as the total protein content. The plasma value of 0.1–0.05 μg Fe/mg protein is higher than the value of 0.015 μg Fe/mg of protein in human plasma (Altman, 1961; Bishop and Surgenor, 1964).

Size exclusion chromatography of the cell lysate showed two main peaks: a high molecular weight protein that elutes at V_o using Sephadex G-75, and a low molecular weight compound, tunichrome (Macara *et al.*, 1979). Iron was found in both peaks, and some iron was eluted after the second peak, probably free iron, because of the high acidity of the eluent.

Chromatography of the plasma resulted in the isolation of a high molecular weight protein; tunichrome was also observed in some cases, probably due to cell lysis during centrifugation. No iron was observed with the high molecular weight protein, however some iron was observed after the tunichrome peak.

In a comparative study on the distribution of metal ions in the plasma of ascidians *Pyura stolonifera* and *Ascidia ceratodes* (Hawkins *et al.*, 1980), results were obtained which relate closely to our experiments. In common with our study, their chromatography experiments show a protein that elutes at V_o, and low molecular weight fractions that test positive for N-acetylaminosugar and negative for protein, and could be assigned to tunichrome. No iron was detected in the high molecular weight fraction. Some iron was found in the low molecular weight fractions when the eluent contained NaCl; however when distilled water was used as an eluent, no iron was detected. Although this result is explained as iron impurities in the NaCl, it is more likely that the iron binds to the gel due to the low ionic strength of the eluent (namely distilled water).

From SDS electrophoresis of the blood cells and plasma there are two protein components in the plasma that are found on the membranes as well (molecular weights 31,000 and 26,000 in the 10% acrylamide gel). These two components are probably obtained from the single denatured high molecular weight protein observed in gel chromatography of plasma proteins. Electrophoresis without SDS of the native plasma proteins yields one major protein band corroborating the chromatography results. Electrophoresis of plasma doped with iron(III)-citrate shows that this protein has an iron affinity high enough to compete with the citrate ligand. No attempt to determine the molecular weight of this ^{55}Fe-labeled protein was made.

Similar iron binding results were obtained by Webb and Chrystal (1981) using blood plasma of the ascidian *Herdmania momus* (order Pleurogona). However, in their experiments the iron(III) was added as iron chloride in 0.1 *N* HCl and then neutralized with bicarbonate. This procedure can cause the iron to precipitate or form high molecular weight iron hydroxide polymers in solution that could be eluted at the exclusion limit along with high molecular weight proteins.

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